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Biochemistry

Asparaginylglucose: Novel type of carbohydrate linkage

(archaebacteria/cell surface glycoprotein)

FELIX WIELAND*, RENATE HEITZER*, AND WOLFRAM SCHAEFER†

*Institut für Biochemie I der Universität Regensburg, Universitätsstrasse 31, D-8400 Regensburg, Federal Republic of Germany; and †Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany

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ABSTRACT **The *Halobacterial* cell wall glycoprotein was recently shown to contain two types of sulfated saccharides: a repetitive saccharide and a nonrepetitive saccharide composed of glucuronic acid and glucose. A new type of *N*-glycosidic linkage is found in this latter type of saccharide: glucose is *N*-glycosidically linked to the polypeptide chain through the amido nitrogen of an asparagine residue, as shown by chemical analyses, proton magnetic resonance spectroscopy, and mass spectroscopy of an isolated asparaginyl saccharide. The only *N*-glycosidic linkage known so far is between the amido nitrogen of asparagine and *N*-acetylglucosamine.**

In glycoproteins, many different types of *O*-glycosidic linkages between sugar residues and amino acids have been described (1). On the other hand, only a single type of *N*-glycosidic bond has been detected so far: *N*-acetylglucosamine, *N*-glycosidically bound to the amido nitrogen of asparagine (2, 3).

The cell wall glycoprotein of the halobacteria, as first described by Mescher and Strominger, possesses both *N*- and *O*-glycosidically linked carbohydrates (4). According to these authors, one high molecular weight polysaccharide, containing neutral hexoses and amino sugars, is presumably attached to asparagine through GlcNAc. Furthermore, about 22 threonine-linked disaccharides containing glucose and galactose and about 12 trisaccharides with an additional unknown uronic acid residue were reported to occur. More recent investigations in our laboratory have revealed that the high molecular weight saccharide of the halobacterial cell-wall glycoprotein has a sulfated repetitive structure similar to animal glycosaminoglycans rather than a heterosaccharide (5). In addition we found sulfated saccharides of low molecular weight that contain glucose and glucuronic acid (6). Our first attempts using β-elimination failed to define the amino acid residue involved in the linkage of these acidic saccharides to the protein. Therefore, we attempted to isolate the corresponding aminoacyl saccharide. Here, we describe the isolation and analysis of an aminoacyl saccharide with the composition 1 Asn/1 Glc/3 GlcUA/3 SO_4^{2-} with glucose as the sugar moiety *N*-glycosidically linked to the amido nitrogen of asparagine.

MATERIALS AND METHODS

General Methods. *Amino acid analyses.* Amino acid analyses were performed after hydrolysis in constant boiling HCl at 105°C for 20 hr as in ref. 7 or by gas/liquid chromatography (GLC) as in ref. 8 in order to determine proline and hydroxyproline. Cysteine was analyzed after treatment of the samples with formic acid/H_2O_2 (9) before hydrolysis. Norleucine served as an internal standard throughout.

Carbohydrate analyses. Neutral sugars were analyzed by GLC as their alditol acetates (10) on a 1-m SP 2340 (Supelco) packed column after hydrolysis in 2 M trifluoroacetic acid at 100°C for various times. Colorimetric estimations were done by the anthrone method (11) with glucose as standard. Uronic acids were determined by either of the following methods: quantitatively by the colorimetric assay (12) with glucuronic acid as the standard; qualitatively by methanolysis (0.5 M HCl in methanol at 80°C for 24 hr) and subsequent gas/liquid chromatography on a Durabond 1701 capillary column (30 m) as their pertrifluoroacetylated methyl glycosides as described (13). Alternatively, their neutral sugar derivatives were prepared by repeated activation/reduction with 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (Serva, Heidelberg) and sodium borohydride as described (14) or by repeated lactonization and reduction with sodium borohydride (15). Galacturonic acid and myoinositol were used as internal standards. Amino sugars were determined on the amino acid analyzer (Durrum 6 A column) as described (7). Glucosamine·HCl and galactosamine·HCl (Sigma) served as standards.

Other Methods. For analytical thin-layer electrophoresis, the samples were dansylated as described (16). Electrophoretic separation was performed on cellulose thin-layer plates (20 × 40 cm, Polygram 400, Machery and Nagel, Düren, Federal Republic of Germany) in pyridine/acetic acid/water, 6:60:934 (vol/vol; pH 3.5), at 2,000 V for 1 hr (first dimension). Development in a second dimension was carried out by chromatography in butanol/pyridine/water, 35:35:30 (vol/vol). The resulting spots were photographed under UV light. Dansylated amino acids were chromatographed on polyamide thin-layer sheets (4 × 4 cm, Schleicher & Schüll, Dassel, Federal Republic of Germany) with the solvents as described (17). Silica thin-layer chromatography (Kieselgel 60, Merck) was performed with butanol/acetic acid/water, 80:20:30 (vol/vol) and evaluated by spraying with ninhydrin (1 mg/ml in ethanol) and orcin [1 mg/ml in 20% (vol/vol) sulfuric acid] reagents. Chromatograms of SO_4^{2-}-labeled samples were fluorographed as described (18). Mass spectroscopy was performed by using the "fast atom bombardment" method (19) on a Finnigan MAT 312 mass spectrometer. Suspensions of ≈20–50 μg of the sample in glycerol were used.

The proton NMR spectrum was determined with a Bruker 300 MHz instrument operated at 300 MHz with the Fourier transform method. About 1.5 μmol of the sample was exchanged by repeated (four times) lyophilization from ^2H_2O (99.96%, Merck Sharp & Dohme, Montreal) and finally dissolved in 250 μl of ^2H_2O.

Glycopeptide Isolation. *Halobacterium halobium* strain M_1 was grown as described (7). Cells were broken by twice freezing in liquid nitrogen a cell suspension in basal salts and thawing. Thereafter cell envelopes were prepared and delipidated as described (4). $^{35}SO_4^{2-}$-labeling and isolation of $^{35}SO_4^{2-}$-labeled gly-

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Abbreviation: GLC, gas/liquid chromatography.

coprotein have been described (7).

Pronase digestion. The protein concentration of the delipidated envelopes was adjusted to 50 mg/ml with 0.1 M Tris·HCl buffer (pH 7.5) containing 10 mM $CaCl_2$, and the sample was incubated with 5 mg of Pronase E per ml (Serva) at 39°C with shaking under toluene. After 12 hr an additional 5 mg of Pronase per ml was added, and incubation was continued for another 12 hr at 56°C. Thereafter, the mixture was passed through a column of Dowex AG 50WX8 H^+ ion-exchange resin (200–400 mesh) in water to remove Pronase and peptide materials. The eluant (about 95% of the radioactivity) was neutralized with solid $NaHCO_3$ and concentrated by rotary evaporation. The resulting solution was chromatographed on Bio-Gel P-10 as described (6). The sulfated oligosaccharide peptides eluted with the included volume of the column.

QUAE-Sephadex chromatography. The radioactive fractions were pooled, diluted with one volume of water, and applied to a QUAE-Sephadex A-25 column equilibrated with 0.15 M ammonium formate buffer (pH 4.0). After the column was washed with five bed volumes of the starting buffer, the column was eluted with a linear gradient (0.15–2.0 M, 12 bed volumes) of the above buffer. Fractions were pooled as indicated in *Results* and dried by lyophilization. To separate residual ammonium formate from the glycopeptides, the dry residue was extracted three times with 50 ml of ethanol. The residue (95% of the radioactivity) was dissolved in water and passed through a short column of Dowex AG 50W H^+ ion-exchange resin in water. The eluant was neutralized with pyridine and concentrated by rotary evaporation.

Preparative high-voltage paper electrophoresis. Amounts of the glycopeptide mixture containing up to 10 mg of uronic acid were loaded on Whatman 3 MM paper and electrophoresed for 150 min at 2,000 V in pyridine/acetic acid/water, 6:60:934 (pH 3.4). After drying, the paper was subjected to autoradiography, and the material was extracted according to the bands on the x-ray film. After passage through a small column of cation-exchange resin in water, the eluant was neutralized with pyridine and dried by rotary evaporation.

High-performance liquid chromatography. For high-performance liquid chromatography, a Waters apparatus was used that was equipped with 6,000 A solvent delivery system and a model 660 solvent programmer. Continuous detection of radioactivity was achieved by using a Berthold flow cell counter. Up to 0.5 μmol of the sample was loaded onto an anion-exchange column [4.5 × 250 mm, Nucleosil 10 $N(CH_3)_2$; Machery and Nagel] and eluted with a linear gradient of pyridinium acetate buffer (pH 5.5) from 0.3 M to 0.9 M in 35 min at a flow rate of 1.5 ml/min. After passage through a small column of cation-exchange resin, fractions containing "material of interest" were lyophilized.

Hydrogen fluoride treatment. Anhydrous hydrogen fluoride (Merck) was condensed into a polyethylene tube cooled by liquid nitrogen with no further care taken to exclude humidity. The frozen hydrogen fluoride then was transferred to an ice bath. Immediately after the acid melted, about 500 μl was pipetted to the sample (20 μg up to 2.5 mg), which had been dried in a plastic microreaction vial. The vial was closed and incubated at 0°C. The reaction was stopped by blowing the hydrogen fluoride into a fume hood by a gentle stream of nitrogen.

Addition of anisol as a scavenger did not lead to a different result and was therefore omitted.

After the treatment the samples were passed through a column (0.5-ml bed volume) of Dowex AG 50W H^+ ion-exchange resin in water and washed with 7 ml of water. Then the column was developed with 3 ml of 8 M ammonia. The eluants were dried by rotary evaporation.

RESULTS

Isolation of an Asparaginyl Saccharide. During recent investigations on the structure of the cell-wall glycoprotein of *H. halobium*, we had obtained a fraction of sulfated glycopeptides of low molecular weight (6). This material contained glucose and glucuronic acid but no glucosamine, and incubation under mild alkaline conditions (50 mM NaOH/1 M $NaBH_4$ at 45°C for 8 hr) to achieve a β-elimination did not liberate significant amounts of carbohydrate. To determine the linkage type within these glycopeptides, we decided to isolate the corresponding aminoacyl saccharide.

Because no difference was observed in the fraction of low molecular weight sulfated glycopeptides between cell envelopes and isolated cell wall glycoprotein [the only glycoprotein in *Halobacteria* (4)], the procedures described below were carried out with $^{35}SO_4^{2-}$-labeled envelopes as the starting material.

The low molecular weight sulfated glycopeptides derived after exhaustive Pronase digestion and subsequent gel filtration (6) were further purified by anion-exchange chromatography on QUAE-Sephadex. The elution profile (Fig. 1) showed three radioactive peaks. Quantification of neutral sugars and uronic acids revealed that both followed the $^{35}SO_4^{2-}$-radioactivity profile. The fractions of the main peak II were pooled as indicated and subjected to preparative high-voltage paper electrophoresis (Fig. 2). One band migrating in front was clearly separated from the residual glycopeptides. The composition of the eluted fractions is given in Table 1. Band I and band II material both contained aspartic acid together with less amounts of glycine, as determined in the amino acid analyzer. In addition, glucose, GlcUA, and sulfate were found. Because it separated best from the bulk of the glycopeptides, band I material was further investigated. Amino acid analysis by GLC showed aspartic acid and glycine in the same ratio as determined in the amino acid analyzer, excluding the occurrence of proline and hydroxyproline. Thus, most probably the sample was an asparaginyl saccharide with a glycopeptide impurity containing aspartic acid and glycine.

For further purification, this sample (Fig. 2, band I) was subjected to high-performance liquid chromatography on an anion-exchange column. The elution profile showed mainly one asymmetric radioactive peak (Fig. 3). Only the top fraction was further investigated. The composition of this material turned out

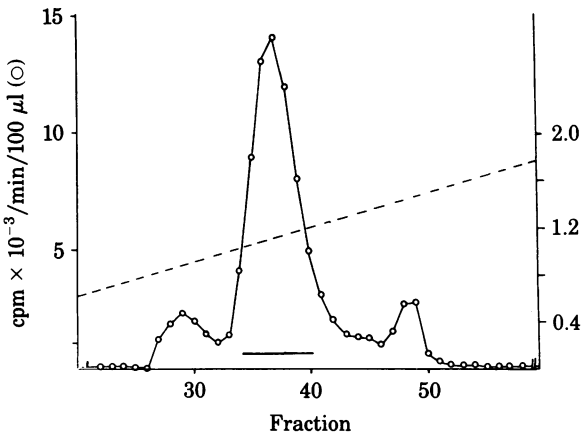

FIG. 1. Separation of $^{35}SO_4^{2-}$-labeled glycopeptides by ion-exchange chromatography. Up to 25 μmol of $^{35}SO_4^{2-}$-labeled oligosaccharide-peptides was chromatographed on a QUAE-Sephadex A 25 column (15 × 2.5 cm) with a linear gradient (---) of ammonium formate buffer (pH 4.0) from 0.15 M to 2 M. Fractions containing 6 ml were collected and pooled as indicated with the bar.

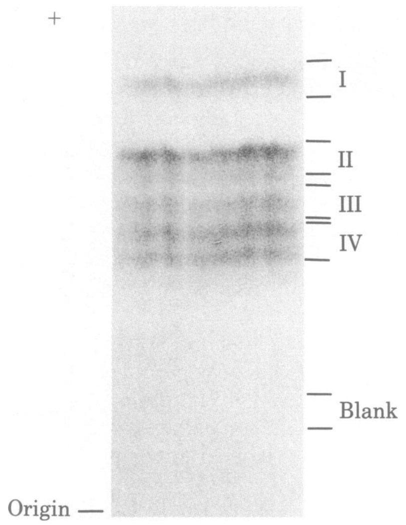

FIG. 2. Autoradiography of a preparative high-voltage electrophoresis. Up to about 10 μmol of the glycopeptide mixture (peak II in Fig. 1) was separated on Whatman 3 MM paper. The electropherogram was subjected to autoradiography, and the material was eluted as indicated.

to be 1 Asp/1.7 NH_3/0.9 Glc/2.5 GlcUA/2.5 SO_4^{2-}. For the quantitative determinations of the carbohydrates, colorimetric assays were used. The results indicated an asparaginyl saccharide of composition 3 GlcUA/3 SO_4^{2-}/1 Glc and were in good agreement with a molecular weight estimation on Bio-Gel P-2 (not shown), where the sample was eluted from the column after the void volume and before the tetrasaccharide stacchiose.

To exclude a (possibly unlabeled) impurity, the material was dansylated and subjected to a two-dimensional separation (Fig. 4). Besides the dansyl free acid, only one spot of fluorescence could be detected, which coincided with the spot obtained after autoradiography of the thin-layer plate, indicating homogeneity of the material. After hydrolysis (6 M HCl for 6 hr at 105°C) the dansylated sample comigrated with dansylated aspartic acid on polyamide sheets in two directions.

Determination of the Linkage Sugar. *Chemical analysis.* The asparaginyl saccharide appeared in the void volume of the amino acid analyzer and, thus, could be quantitated easily (Fig. 5*A*, trace a). This allowed us to follow the effect of different conditions for partial hydrolysis. Incubation with 2 M trifluoroacetic acid or 0.5 M HCl for various times at 100°C gave rise to various amounts of aspartic acid, but almost no intermediate product was found. One method to discriminate between *N*- and *O*-glycosidic bonds is hydrolysis in anhydrous hydrogen fluoride (20). After incubation for 3 hr at 0°C in condensed hydrogen fluoride, about 70% of the starting material was lost, and a new peak appeared in the analyzer, eluting 8 min before aspartic acid (Fig. 5*A*, trace b). To isolate this product, the $^{35}SO_4^{2-}$-labeled asparaginyl saccharide was treated with hydrogen fluoride, and the products were separated on a cation-exchange column. After the column was washed with water, all radioactivity, but only about 20% of the aspartic acid, was found in the eluant. The column was eluted with ammonia in water,

Table 1. Composition of the fractions obtained after high-voltage paper electrophoresis

Fraction	Asp	Thr	Ser	Glu	Gly	Glc	GlcUA	SO_4^{2-}
I	10.0	—	—	—	1.7	12.9	29.5	34.2
II	10.0	—	—	—	3.9	17.1	33.0	31.5
III	10.0	3.3	2.5	1.3	3.4	14.3	27.3	23.5
IV	10.0	3.9	4.1	3.3	5.8	14.0	27.0	21.6
Blank	—	—	—	—	0.3	—	0.7	—
I*	10.0	—	—	—	0.4	9.4	25.2	25.4
I†	10.0	—	—	—	0.4	8.3	0.6	—

Values are expressed relative to Asp. Sample amounts for the blank determinations were used corresponding to the areas of paper eluted.
* Fraction I after purification by high-performance liquid chromatography.
† Fraction I after high-performance liquid chromatography, hydrogen fluoride treatment, and elution from cation-exchange resin.

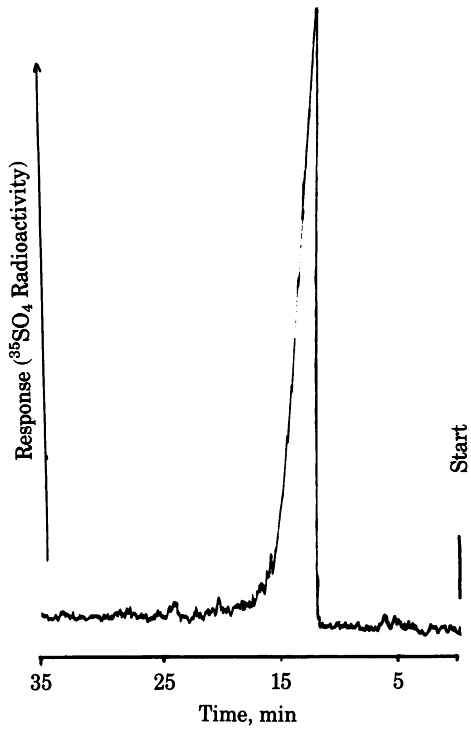

FIG. 3. High-performance liquid chromatography. Fraction I (about 500 μg; 6 × 10^5 cpm) was chromatographed on an anion-exchange column [$N(CH_3)_2$; 4.5 × 250 mm]. After washing with 10 bed volumes of starting buffer, a gradient was applied from 0.34 M to 0.9 M of pyridinium acetate buffer (pH 5.5). The run was for 35 min at room temperature with a flow rate of 1.5 ml/min (1,800 psi; 1 psi = 6.89 kPa), and 0.75-ml fractions were collected. The radioactivity was monitored continuously by a flow cell counter.

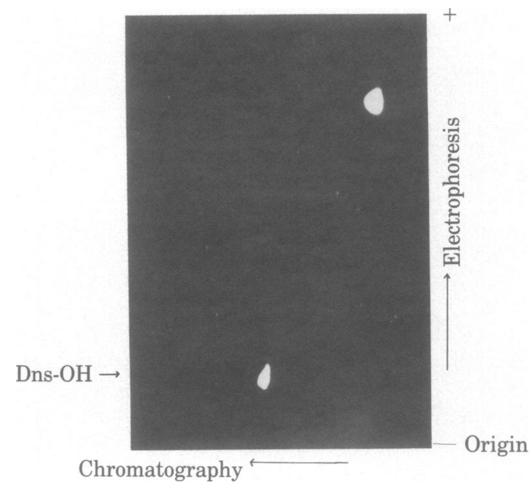

FIG. 4. Two-dimensional separation of the asparaginyl saccharide. The sample (top fraction, Fig. 3) was dansylated and subjected to high-voltage thin-layer electrophoresis (first direction) and chromatography (second direction). The thin-layer sheet was photographed under UV light. Dns-OH, dansyl free acid.

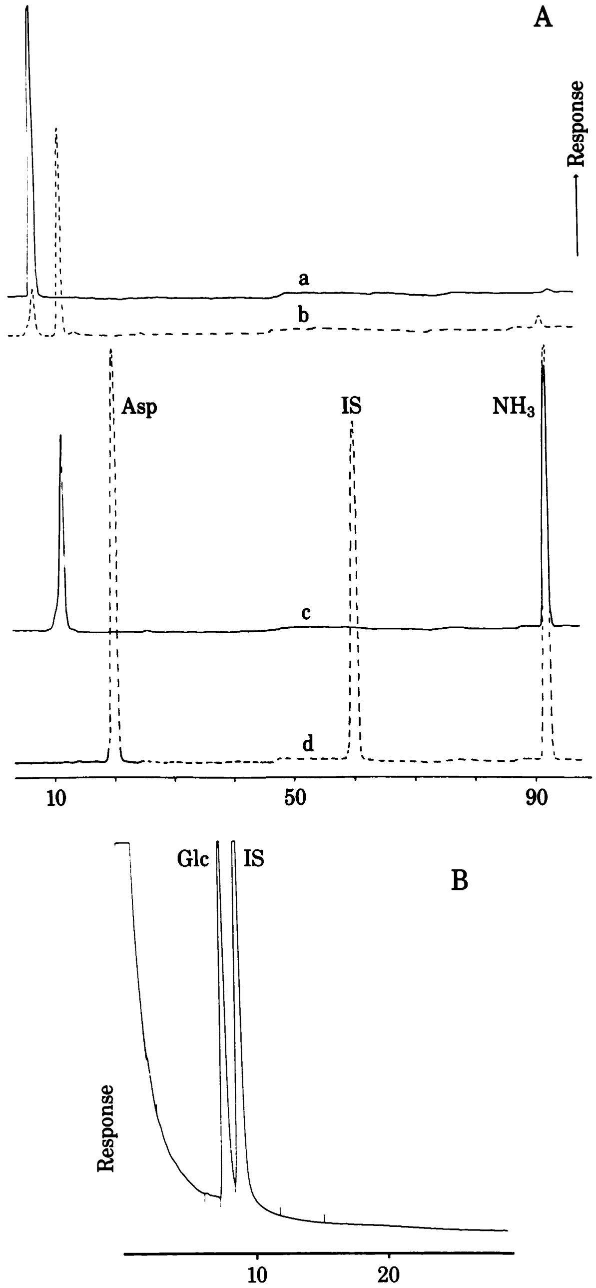

FIG. 5. Analysis of the products after hydrogen fluoride treatment. (*A*) On the amino acid analyzer. Traces: a, untreated sample; b, after incubation with hydrogen fluoride for 3 hr at 0°C; c, after hydrogen fluoride treatment, adsorption on cation exchange resin, and subsequent elution with 8 M NH_3; d, sample as in trace c after hydrolysis in constant boiling HCl at 100°C for 6 hr. IS, internal standard (norleucine). (*B*) GLC. The sample in *A*, track c, after hydrolysis (2 M trifluoroacetic acid for 12 hr at 100°C), reduction, and peracetylation, was chromatographed on a packed column (1 m) of SP 2340. Conditions for the run: carrier gas (N_2) at 30 ml/min and temperature gradient from 200 to 240°C at 2°C/min. IS, internal standard (myoinositol).

and an aliquot was assayed in the amino acid analyzer. Only the peak appearing 8 min before the aspartic acid peak was observed (Fig. 5*A*, trace c). The sample migrated as a single spot on a silica gel thin-layer plate. Amino acid analysis together with the colorimetric anthrone assay revealed a stoichiometry of 1.2 Asp/1.0 hexose (Fig. 5*A*, trace d). After hydrolysis (2 M trifluoroacetic acid for 12 hr at 100°C), reduction, and peracetylation, glucitol was found by GLC to be the only component at

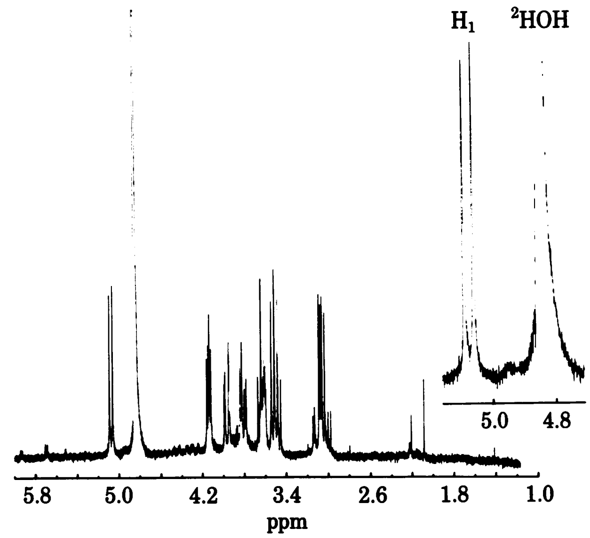

FIG. 6. Proton-magnetic resonance spectrum (300 MHz) of the isolated compound asparaginylglucose.

a ratio to aspartic acid that was exactly the same as found with the anthrone assay (Fig. 5*B*). An aliquot of the hydrolyzed sample was assayed enzymatically for D-glucose as described (21). This test showed that the hexose present in the isolated asparaginyl saccharide is D-glucose.

Mass and proton magnetic resonance spectroscopy. The product was further investigated by "fast atom bombardment" in the mass spectrometer as described (19). With the free acid, the highest mass peak found was 295, in agreement with a protonated compound asparaginylglucose. After neutralization with $NaHCO_3$, the highest mass peak was 317, corresponding to the sodium salt of asparaginylglucose.

To obtain a proton magnetic resonance spectrum of the compound, about 2.5 μmol of the asparaginyl saccharide after elution from the electropherogram (band I) was treated with hydrogen fluoride. Amino acid analysis of the fraction eluting from the cation exchange resin with NH_3/water revealed a glycine impurity of 12–15%. The yield was about 0.5 mg. In Fig. 6 the proton magnetic resonance spectrum is shown. The C_1-hydrogen of glucose appears as a doublet at 5.09 and 5.06 ppm (relative to ^{2}HOH set to 4.85 ppm), with a coupling constant $J_{1,2}$ of 9.2 Hz. This value is in good agreement with that for β_1-asparaginyl-GlcNAc (22), indicating a β-glycosidic linkage.

DISCUSSION

The results reported here clearly show that the acidic saccharide is bonded to an asparagine residue of the protein through a glucosyl residue in β-linkage. So far only the asparaginyl saccharide has been purified to homogeneity. The composition of the partially purified glycopeptides (compare Table 1) suggests, however, that all the sulfated saccharides have the same linkage type because (*i*) aspartic acid is the predominant amino acid found after hydrolysis in each of the fractions, (*ii*) no sugar was released under mild β-elimination conditions [0.05 M NaOH/1 M $NaBH_4$ at 45°C for 8 hr (23)], and (*iii*) the fractions differ principally in their peptide composition and the degree of sulfation and to a lesser extent in their carbohydrate composition. Two findings make it highly likely that the glucose residue is linked to the β-amino nitrogen of asparagine: (*i*) at least 10 mol of sulfated oligosaccharides occur within 1 mol of glycoprotein, based on an average molecular weight of 1,000 for the oligosaccharides, whereas only one α-amide per mol of glycoprotein of aspartic acid can occur, namely at the COOH-terminus of the protein chain, and (*ii*) the compound asparaginylglucose is eluted from the amino acid analyzer before aspartic acid. If the more acidic α-carboxyl group (pK ≈ 2.0) were blocked, the resulting compound would have a pK of about 3.9 (β-carboxyl of aspartic acid) and consequently would be more retarded on the analyzer. Such effects have been described with peptides containing α- or γ-glutamic acid residues (24).

We have not yet found conditions to hydrolyze the sulfate residues from the carbohydrate without partial destruction of the saccharides. However, the 1:1 ratio of $GlcUA/SO_4^{2-}$ strongly suggests that the sulfate residues are bound to the glucuronic acids. The inability to specifically hydrolyze the sulfate groups has hampered us so far in our attempts to determine the linkage between the carbohydrate residues, because under the strong alkaline conditions necessary for permethylation analysis, a migration of the sulfate residues cannot be excluded.

The existence of a threonine- or serine-type *O*-glycosidic linkage is often inferred from β-elimination studies. Recently a partial degradation of *N*-glycosidic linkages has been shown to occur by prolonged incubations under mild alkaline conditions (25, 26). With our finding of a second type of *N*-glycosidic linkage, one could expect that a critical reevaluation of such experiments might reveal new types of *N*-glycosidically linked sugars in a variety of organisms.

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